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PAPER

Suppression of experimental systemic lupus erythematosus (SLE) in mice via TNF inhibition by an anti-TNFα monoclonal antibody and by pentoxiphylline

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We have previously shown that the clinical manifestations of experimental systemic lupus erythematosus (SLE) correlate with an early increased secretion of TNFα and IL-1. In the present study, we examined the efficacy of two therapeutic modalities which lower TNFα production or activity, on the clinical manifestations of the disease. Experimental SLE was induced in naive C3H.SW mice by injection of the human anti-DNA monoclonal antibody (mAb) bearing the common idiotype, 16/6 Id. Two weeks after booster injections, treatment with either an anti-TNFα mAb, or pentoxiphylline (PTX) was started, for a period of 6 weeks. Production of TNFα (by splenocytes) and IL-1 (by peritoneal macrophages) was determined 3 and 7 months after disease induction. The experimental mice were also followed for disease manifestations. Both treatment protocols, with anti-TNFα mAb and with PTX, reduced the production of the two pro-inflammatory cytokines, TNFα and IL-1, in mice with experimental SLE. Anti-DNA antibodies were significantly lower in the mice treated with either protocol. In addition, a significantly lower rate of leukopenia, proteinuria and immune complex deposition was observed in treated mice. Abrogation of TNFα and IL-1 production in the early stages of experimental SLE by an anti-TNFα mAb or by PTX improves the clinical status of mice afflicted with this autoimmune disease. *Lupus* (2001) **10**, 23–31.

Keywords: anti TNF; experimental SLE

Introduction

Experimental systemic lupus erythematosus (SLE) is induced in naive C3H.SW mice that do not develop SLE spontaneously, by immunization with a human monoclonal anti-DNA antibody that expresses a common idiotype (Id) designated 16/6 Id. After immunization with the 16/6 Id, all the mice develop high titers of antibodies specific to DNA, and to 16/6 Id, as well as SLE-related clinical manifestations including leukopenia, thrombocytopenia, proteinuria and immune complex glomerulonephritis.[1]

A significant early elevation of TNFα and IL-1 production was observed at an early stage of the disease, followed by a cascade of Th_1- and later Th_2-type cytokines. The increased production of these pro-inflammatory cytokines persisted for at least 6 months (throughout the duration of the experiment).[2]

To further support the role of the proinflammatory cytokines in the model of experimental SLE we have previously shown that other therapeutic modalities that were clinically beneficial (methotrexate and tamoxifen) significantly abrogated the early increased production of IL-1 and TNFα.[3–5]

The important role of TNFα (and IL-1) in the pathogenesis of SLE has been postulated in several studies, both in humans[6–9] and in murine SLE models,[10–14] and therefore inhibition of its production or activity seems to be a logical therapeutic modality.

In order to find out whether the reduced levels of TNFα will have beneficial effects on SLE manifestations, we have examined the therapeutic effects of two anti-TNF modalities in mice with experimental SLE. Thus we administered either anti-TNFα monclonal antibody (mAb) or pentoxiphylline (PTX), a drug which has anti-TNF properties, to mice with 16/6 Id-induced

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experimental SLE. We demonstrate here beneficial effects on the clinical manifestations of lupus, which were accompanied by a significant reduction in the production of the pro-inflammatory cytokines, IL-1 and TNFα, with both therapeutic protocols.

Materials and methods

Mice

Female C3H.SW mice from the Jackson Laboratory (Bar-Harbor, Maine, USA) were used throughout the study.

Antibodies and antigens

The human monoclonal anti-DNA antibody, bearing the 16/6 Id was isolated on a protein G-Sepharose column (Pharmacia) from culture supernatants of the hybridoma secreting the antibody.[2] The 16/6 mAb was initially of the IgM isotype but switched to IgG1 while it was growing in culture. The mAb of the IgG1 isotype was found to be as effective as that of the IgM isotype at inducing experimental SLE.[15]

Induction of experimental SLE

The immunization protocol to induce the SLE-like disease was as previously described.[3] Briefly, mice were immunized intradermally into the hind footpads with 1 μg of the 16/6 mAb, in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit MI, USA). Three weeks later, booster injections, with the same amount of antibody in phosphate buffered saline (PBS), were given at the same sites. The mice were bled and their sera were tested for antibodies of various specificities, starting 3 weeks following the booster injection.

Treatment protocols

Mice were injected intraperitoneally (i.p.) with 50 μg, anti-TNFα mAb, twice weekly for a period of 6 weeks. This is a rat anti-mouse mAb V1q, which was purified from cell culture supernatant using Protein G column chromatography. This antibody reacts with mouse TNF.[16] PTX (Hoechst Pharmaceuticals) was also injected i.p., 100 μg daily for 6 weeks. Control groups immunized with 16/6 Id received i.p. injections with saline.

Assays of cytokine production

Assays of cytokine production by macrophages and lymphocytes were as in our previous studies.[2]

Peritoneal macrophages were washed with cold PBS supplemented with fetal calf serum (FCS) and pooled from mice in each experimental group. The cells were then incubated (10^6/ml) with, or without lipopolysaccharide (LPS; 10 μg/ml), in enriched culture medium consisting of RPMI supplemented with 10% FCS for 24 h. Supernatants were tested for the production of IL-1. Splenocytes (5×10^6/ml) pooled from mice in each experimental group, were incubated with or without concanavalin A (Con A; 2.5 μg/ml) in culture medium for 24 h. Supernatants were tested for the presence of TNFα. IL-1 and TNFα levels were tested by ELISA using the relevant capture and detecting antibodies, as previously described.[2] The results were measured using an ELISA reader at 414 nm.

Assay of antibodies

Assays were performed as previously described.[1,2] Briefly, Maxisorb plates (Nunc, Denmark), coated with either DNA or the 16/6 Id antibody, were blocked with 1% ovalbumin (OVA, Sigma) and incubated with the serum samples. The plates were then washed and incubated with goat anti-mouse IgG (γ chain specific) conjugated to peroxidase (Jackson Immuno Research, PA). Subsequently, the plates were incubated with the substrate, ABTS (2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid); Sigma) and data were recorded using an ELISA reader at 414 nm.

Detection of SLE-associated manifestations

The leukocyte counts were determined by mixing heparinized blood with 1% acetic acid at a ratio of 1 : 10. Proteinuria was measured by a semi-quantitative manner, using a Combistix kit (Ames-Miles, Slough, UK).

Immunohistology

Kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 6 μm

were air dried and fixed in acetone. For the detection of immunoglobulin deposits, sections were incubated with FITC-conjugated rabbit anti-mouse IgG (γ chain-specific; Jackson). Specific staining was visualized using a fluoresence microscope.

Data analysis

Statistical analysis was based on one-way ANOVA comparing the data on cytokine levels, MANOVA for the levels of antibodies during the study period, and

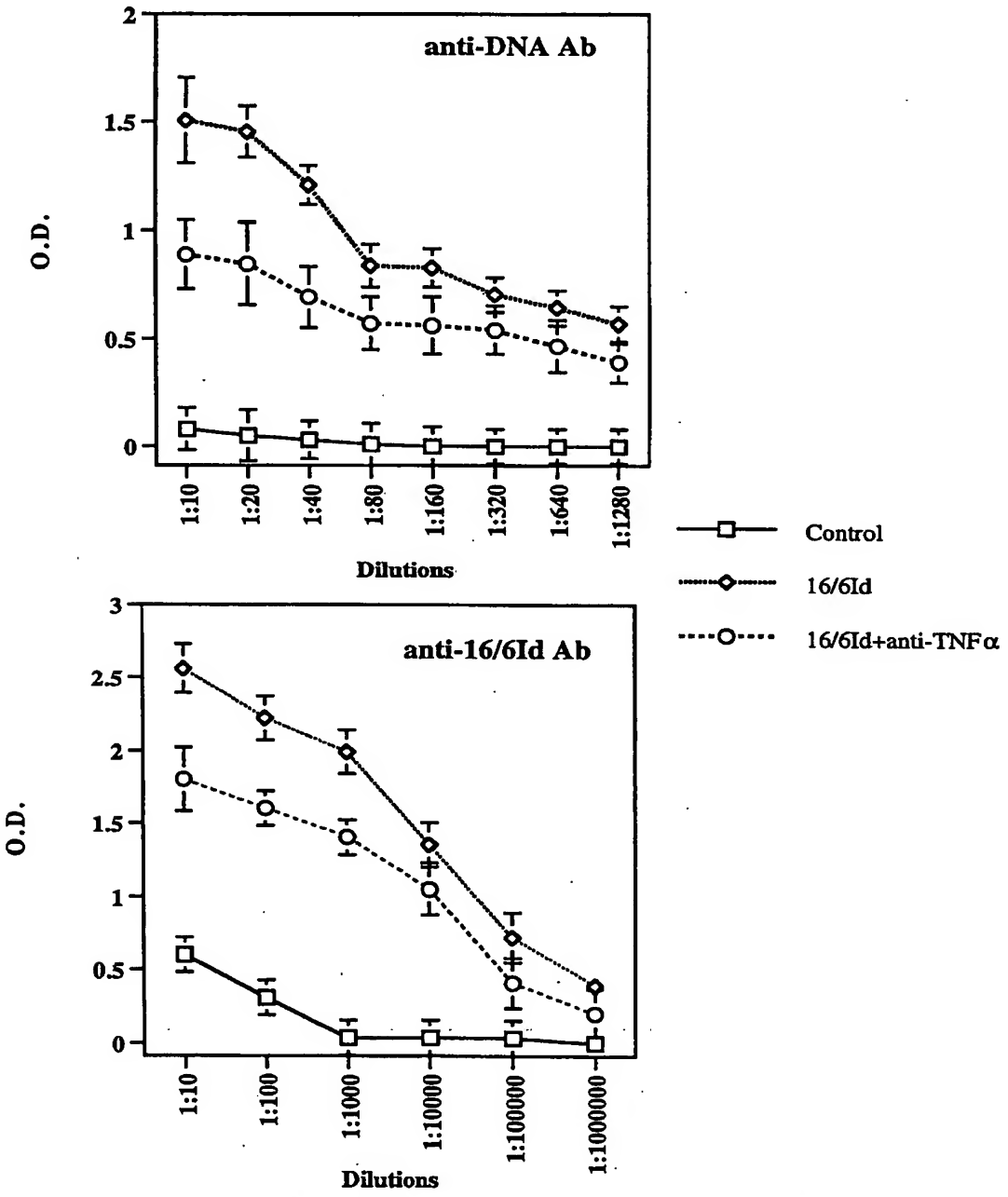

Figure 1 Effects of an anti-TNFα mAb treatment on antibodies to DNA (A) and 16/6 Id (B) in mice with experimental SLE. Results represent the mean of OD obtained from five or six mice in each group, 3 months following immunization with 16/6 Id, and 1 month after ending the treatment. Mice immunized with 16/6 Id and treated with anti-TNF mAb had lower levels of anti-DNA antibodies compared to the immunized and non-treated mice ($P < 0.01$ by MANOVA).

Fisher's exact test for evaluation of the effects on the clinical manifestations.

Results

C3H.SW female mice, at the age of 2 months, were immunized and boosted with the 16/6 Id. Two separate therapeutic trials have been performed. In one, mice were treated with a monoclonal anti-TNFα antibody, starting 2 weeks after booster with the 16/6 Id. Anti-TNFα mAb (50 μg per mouse) was injected i.p. twice a week for a period of 6 weeks.

In the second experimental type, we used PTX as the therapeutic modality. Mice were injected with 100 μg of PTX i.p. daily for 6 weeks, starting 2 weeks after boost. Two independent experiments were performed with each therapautic protocol to confirm the results.

Effects of anti-TNFα and PTX treatments on antibody levels

Figure 1 demonstrates the antibody levels of one representative assay, performed on sera obtained from mice, 3 months following immunization. Immunized mice with 16/6 Id had significantly higher levels of anti 16/6 Id and anti-DNA antibodies, as compared to controls ($P < 0.001$ by MANOVA). The levels of anti-DNA Abs in the mice treated with anti-TNF were significantly lower than those of the 16/6 Id immunized untreated mice ($P < 0.01$ by

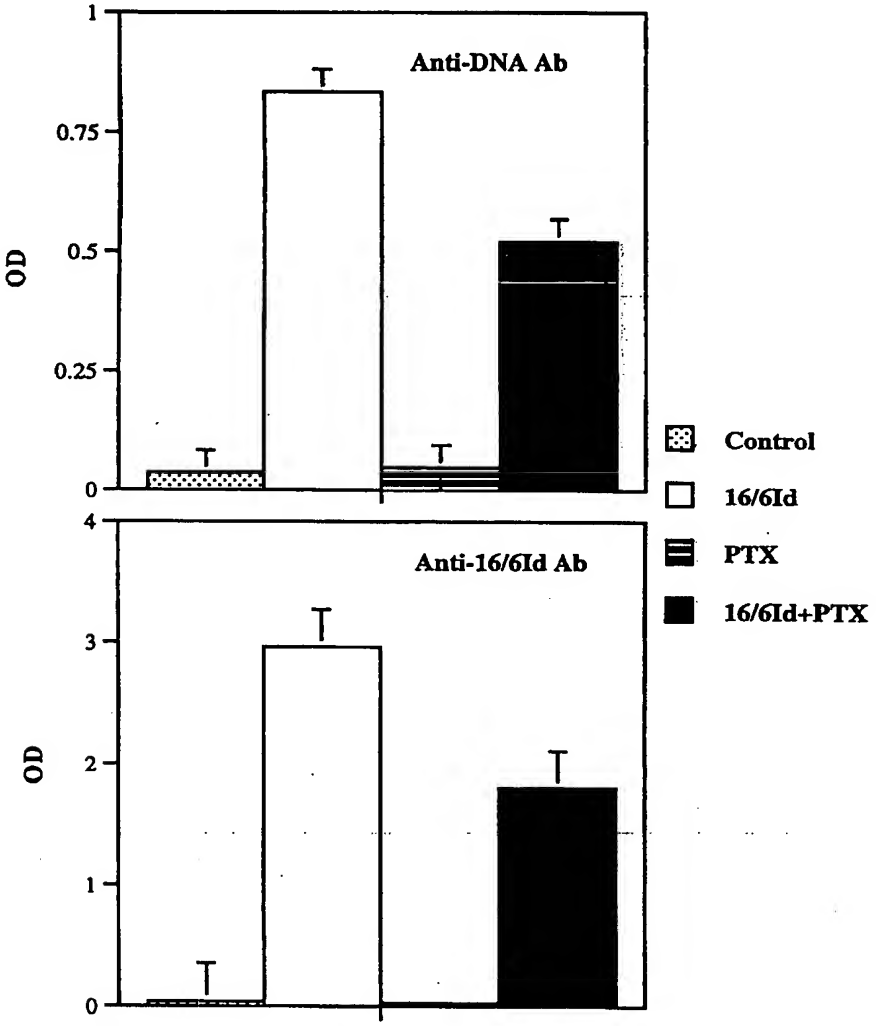

Figure 2 Effects of PTX on antibody levels in mice with experimental SLE. Sera were obtained from five or six mice of each group 3 months following immunization with 16/6 Id, and 1 month after ending PTX treatment. Both anti-DNA and anti 16/6 Id Abs were lower in the immunized and PTX-treated mice, as compared to the immunized nontreated group ($P < 0.001$ by ANOVA).

MANOVA). The levels of anti 16/6 Id specific antibodies were slightly but not significantly reduced following treatment with anti-TNF.

The effects of PTX treatment on antibody levels in the mice with experimental SLE are shown in Figure 2. The mice, which were immunized with the 16/6 Id and treated with PTX, had significantly lower levels of antibodies to 16/6 Id and to DNA ($P < 0.001$ by ANOVA).

The same pattern of antibody profiles was observed when sera of mice were examined at more advanced stages of the disease. Similar results were obtained in the two experiments performed.

Effect of anti-TNFα and PTX treatment on the production of pro-inflammatory cytokines

Since the pro-inflammatory cytokines IL-1 and TNFα are the first to be detected in the cytokine cascade in experimental SLE,[2] we have followed their production at two periods following immunization; 3 and 7 months. As can be seen in Figures 3 and 4, which

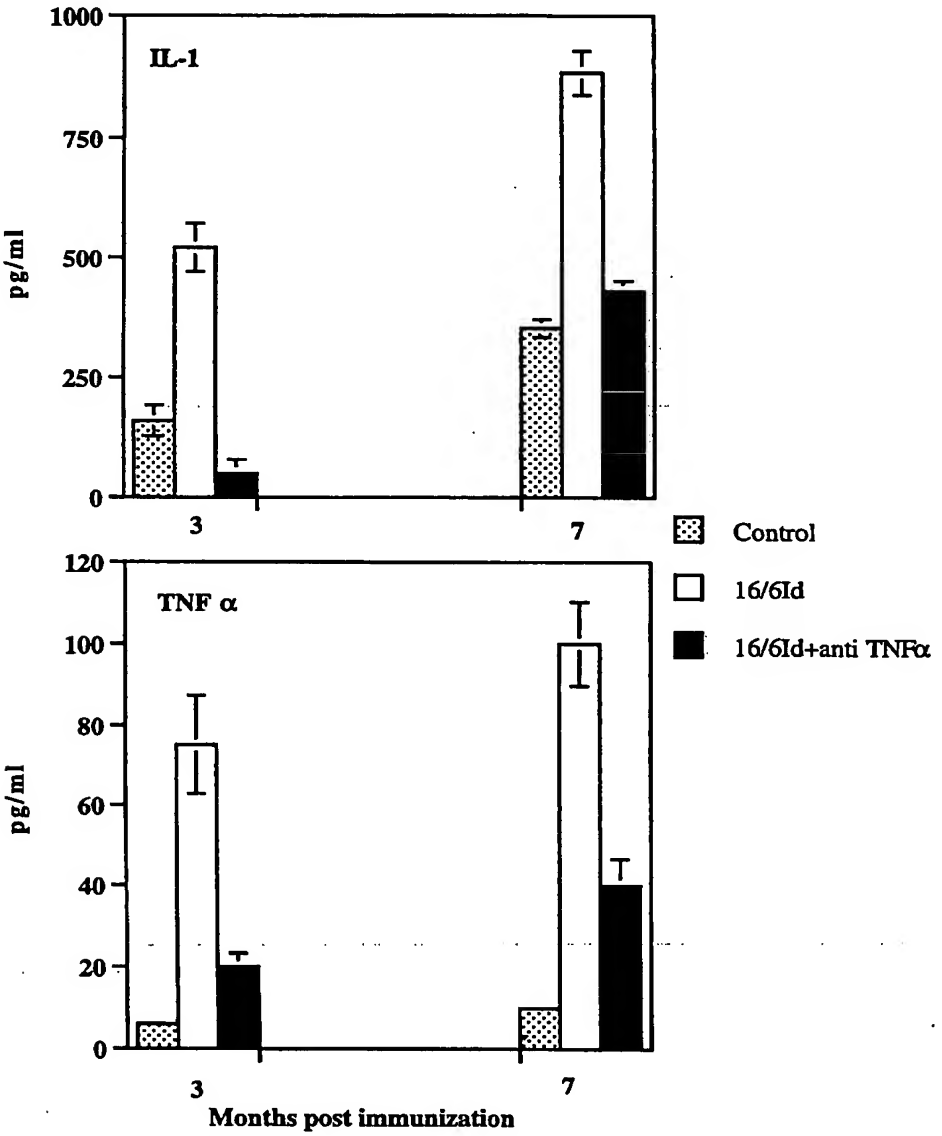

Figure 3 Effects of an anti-TNF mAb treatment on the production of pro-inflammatory cytokines. Production of IL-1 by peritoneal macrophages and of TNFα by splenocytes was measured 3 and 7 months following immunization with the 16/6 Id. Results represent the mean ± s.d. levels of IL-1 and TNFα obtained from 4-6 mice in each group. Anti TNFα mAb treatment significantly reduced the levels of both IL-1 and TNFα ($P < 0.001$ ANOVA).

represent one out of two experiments with each treatment, mice immunized with the 16/6 Id produce high levels of both IL-1 and TNFα, as compared to the control group at both periods tested. Both treatment protocols, anti-TNFα mAb (Figure 3) and PTX (Figure 4), significantly reduced the levels of the two pro-inflammatory cytokines IL-1 and TNFα ($P < 0.001$ by ANOVA). Interestingly, anti-TNFα treatment in mice with experimental SLE was equally effective on IL-1 production as on TNFα itself. In general, the levels of IL-1 and TNFα in the treated mice were reversed to levels close to those observed in the control groups.

Similar results were obtained in the second experiment. Thus levels of TNFα produced by splenocytes obtained from immunized and treated mice 3 months following booster injections were reduced to 40 and 20% of the levels determined for 16/6 Id immunized and untreated mice for the anti-TNFα and PTX treated mice, respectively. Similiarly, IL-1 production by peritoneal macrophages of the same mice was reduced to 20 and 30% respectively.

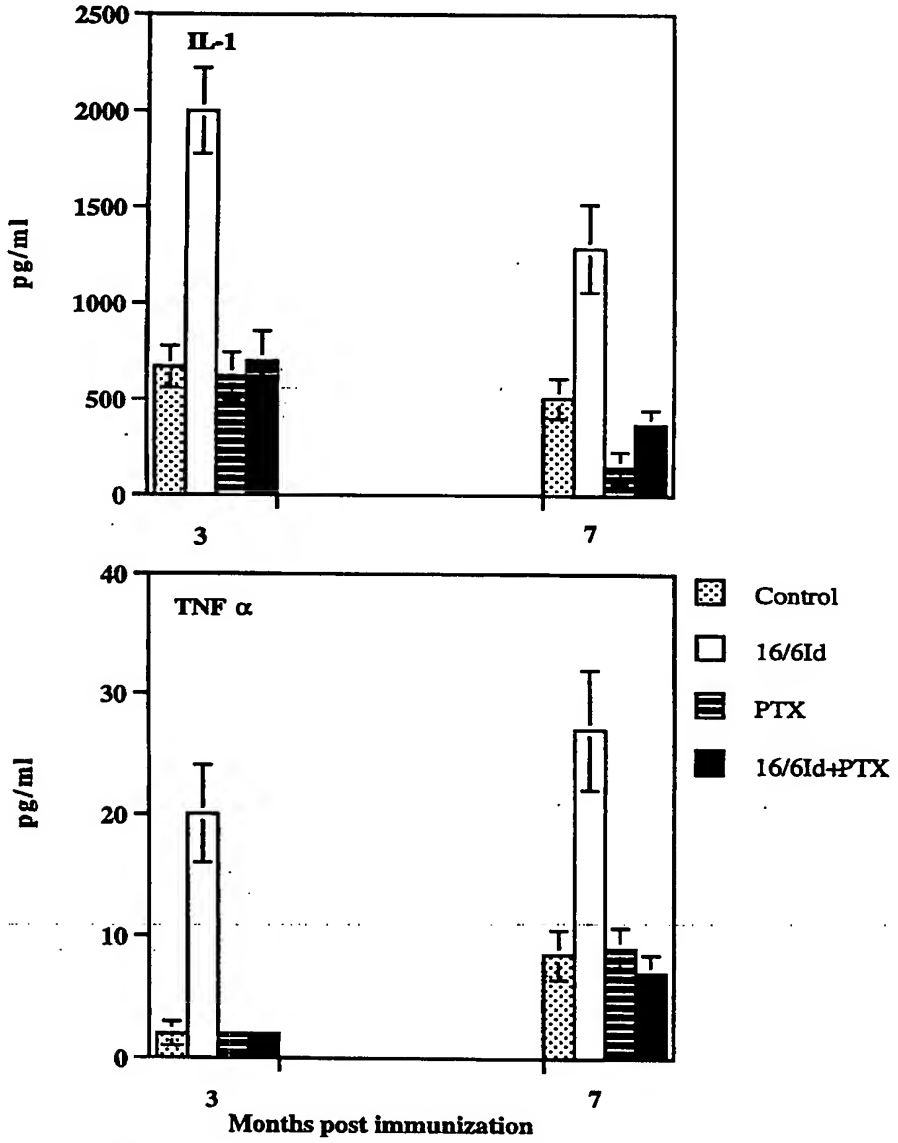

Figure 4 Effects of PTX treatment on the production of pro-inflammatory cytokines. PTX treatment reduced significantly the levels of both IL-1 and TNFα ($P < 0.001$ ANOVA). Other details as in Figure 3.

Table 1 Effect of an anti-TNFα mAb and pentoxiphylline (PTX) treatment on the clinical manifestations of 16/6 Id immunized C3H.SW mice

Treatment group	WBC (mean ± s.d.)	Proteinuria[a] Positive/group	%	Immune complex deposits[b] Positive/group	%
Anti-TNFα mAb					
Control	7300 ± 1100	1/6	17	1/6	17
16/6 Id	3950 ± 1300	5/6	83	5/6	83
16/6 Id + anti-TNFα mAb	6850 ± 900*	2/6	33	1/6*	17
Pentoxiphylline					
Control	7800 ± 1400	2/5	33	1/5	20
16/6 Id	3650 ± 1100	6/6	100	5/6	83
PTX	6700 ± 1200	2/6	33	2/6	33
16/6 Id + PTX	6800 ± 800*	2/6*	33	2/6	33

[a]Number and percentage of mice with proteinuria of more than 0.3 g/L.
[b]Number and percentage of mice with immune complex deposits.
Statistical analysis by student's *t*-test or Fisher's exact test (one tail) while compared to the 16/6 Id immunized and nontreated group; $*P < 0.05$.

Effects on clinical manifestations

Mice were periodically examined for the typical clinical manifestations of the experimental disease. Representative results of these studies are shown in Table 1. Part A demonstrates results of treatment with anti TNFα and the results of PTX therapy are presented in part B. While most mice with the experimental disease show leukopenia, both treatments reversed this effect. Similarly, the presence of proteinuria was lower in both treated groups ($P = 0.06$ and $P = 0.03$ by Fisher's exact test for anti-TNFα and PTX treatment, respectively). Renal damage, manifested by deposition of immune complexes in the kidneys of SLE afflicted mice is one of the major characteristics of experimental SLE. About 80–100% of mice immunized with the 16/6 Id in different studies show immune complex deposits in their kidneys. In the present experiments we have seen that the incidence of renal immune complex deposits was lower in both treatment groups, however it reached significance only in the group treated with the anti-TNFα mAb ($P = 0.04$ and $P = 0.06$ for anti-TNFα and PTX treatment, respectively). Figure 5 demonstrates immunohistology of representative kidney sections of 16/6 Id immunized and untreated mice and of mice treated with either anti-TNFα or PTX. The differences in the intensity of immune complex deposits between treated and untreated mice are shown in the figure. Because the 16/6 Id immunized mice survive for at least 12

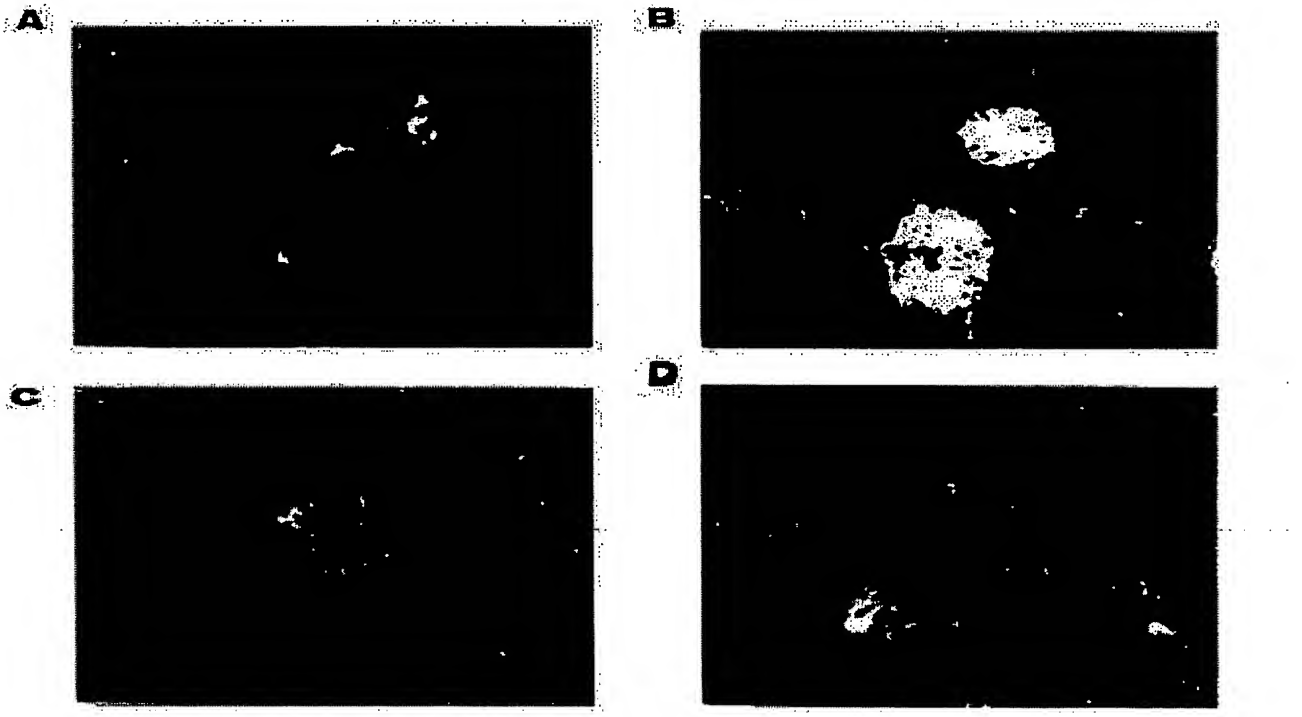

Figure 5 Immunohistology of kidney sections 7 months after immunization with the human 16/6 Id. (A) Control mice. (B) Mice immunized with 16/6 Id. (C) Mice immunized with 16/6 Id and treated with anti-TNFα mAb. (D) Mice immunized with 16/6 Id and treated with pentoxiphylline. Immunofluorescent staining with FITC-labeled goat anti-mouse antibodies of 6 μm frozen sections (×400).

months following disease induction, and they were sacrificed between 3 and 8 months after immunization for assessment of cytokine production and renal manifestations, the effect of treatment on the survival rate was not evaluated in this study.

Discussion

We have shown in this study that decreasing the production of TNFα in early stages of murine experimental SLE has a beneficial effect on the manifestations of the disease. The improvement in the clinical manifestations, for example proteinuria and glomerular immune complex deposition, was accompanied by a decrease in anti-DNA antibodies. The production of IL-1 was significantly reduced concomitant with the reduction in TNFα production.

Two approaches were used in the present study to inhibit TNFα production, of which the first was injections of anti-TNFα mAb. The information concerning the effect of anti-TNFα on lupus is very limited and is restricted to articular and pulmonary manifestations in MRL/lpr/lpr mice.[10,17]

As a second approach we used PTX, a drug with vasoactive activity which was found to possess anti-TNFα properties. The inhibition of TNF gene transcription by this drug is through its effect on phosphordiesterase.[18,19] Additional beneficial effects of PTX have been suggested, such as attenuation of neutrophil activation[20] anti-proliferative activities, shown *in vitro* on B and T lymphocytes,[21] and reduction of the production of oxygen-free radicals.[22] Due to its varied anti-inflammatory and immunomodulatory effects, PTX has been used in several autoimmune diseases in humans: vasculitis, scleroderma, rheumatoid arthritis, Behcet disease, multiple sclerosis, juvenile diabetes mellitus, and even in SLE patients.[23–27]

PTX-treated MRL/lpr mice showed diminished titers of anti-DNA antibodies, decreased proteinuria, and increased survival rate. These beneficial effects correlated with a decreased TNFα production.[28]

We have shown in this study that both treatment protocols were almost as efficient in the inhibition of the production of the two proinflammatory cytokines. The anti IL-1 effect of a drug which is expected to inhibit TNFα is not surprising since TNFα is one of the known factors that augment IL-1 synthesis.

Most murine lupus strains except (NZB×NZW)F1 are characterized by increased TNFα production.[29] In MRL/lpr mice a progressively increased expression of circulating TNFα is proportional to the severity of renal disease.[30] Even in (NZB×NZW)F1, the pathogenic role of TNF is complicated and dichotomous results are available. In certain circumstances, administration of TNFα may have detrimental effects.[31] Moreover, expression of TNFα is increased in the kidneys of MRL/lpr mice,[14,32] as well as in (NZB×NZW)F1 mice.[31,33]

We have shown in several previous studies an early and prominent increased production of TNFα and IL-1, in mice with the 16/6 Id-induced experimental SLE.[2,4] Once induced, these cytokines act as proinflammatory effectors, initiating a cascade of cytokines: Th1-type (IL-2, INFγ) first, and Th2-type (IL-4, IL-10) cytokines later, thus leading to a persistent immune stimulation and progression of the disease.[2,4]

Several studies have shown that human lupus is characterized by high serum levels of TNFα and soluble TNF receptor that parallel disease activity.[6–9,34] Increased expression of TNFα (and IL-6) was found in 52% of kidney specimens of patients with lupus nephritis.[8] Thus, TNFα is suggested to be a major final mediator of many systemic and local inflammatory phenomena, which may lead to the glomerular damage in SLE.[10,12–14]

The role of IL-1 in the pathogenesis of SLE is also based on animal models and lupus patients. Markedly increased expression of IL-1 mRNA was found in glomerular macrophages of MRL/lpr and (NZB×NZW)F1 mice,[14,31,35] in the spleens of mice with graft-*vs*-host-disease-induced lupus,[36] and in peripheral blood mononuclear cells from patients with active lupus.[37,38] *In vivo* effects of IL-1 in (NZB×NZW)F1 mice were noted as well. Thus, administration of low-dose recombinant (r) IL-1a accelerated renal injury and mortality,[31] and rIL-1β accelerated the onset of arthritis in MRL/lpr mice.[39]

In conclusion, we have shown in this study that two therapeutic protocols based on down-regulating TNFα production, namely treatment with an anti-TNFα mAb and PTX, had beneficial effects on the pathological manifestations of experimental SLE in mice. The amelioration of the clinical signs correlated with a significantly abrogated production of both proinflammatory cytokines, TNFα and IL-1.

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